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Metformin regulates tight junction of intestinal epithelial cells via MLCK-MLC signaling pathway

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Abstract. – OBJECTIVE: To observe the effect of metformin on the tight junction of intestinal epithelial cells and its relevant mechanism.

MATERIALS AND METHODS: Caco-2 cell monolayers were incubated with or without tumor necrosis factor-α (TNF-α) (10 ng/mL) in the absence or presence of indicated concentrations of metformin. Transepithelial electrical resistance (TEER) was measured at various time points. Caco-2 cell permeability was assessed using fluorescein permeability test. Immunofluorescence was used to detect the distribution of tight junction protein. Western blotting and Real-Time PCR were used to detect the expression of tight junction protein and Myosin light chain kinase (MLCK)-Myosin light chain (MLC) signaling pathway.

RESULTS: Metformin attenuates the effects of TNF-α on Caco-2 cell TEER and paracellular permeability, prevents TNF-α-induced morphological disruption of tight junctions, ameliorates the inhibiting effect of TNF-α on epithelial tight junction-related protein expression and suppresses the TNF-α-induced increase in MLCK production.

CONCLUSIONS: Metformin can stabilize and up-regulate tight junction protein by inhibiting MLCK-MLC signaling pathway, thus ameliorating the tight junction of intestinal epithelial cells.

Key Words:

Irritable bowel syndrome, Metformin, Intestinal epithelium, Tight junction, MLCK-MLC signaling pathway.

Introduction

Irritable bowel syndrome (IBS) is mainly manifested by abdominal pain or discomfort, which can be ameliorated after defecation. It is often accompanied by defecation habit change and lack of morphological and biochemical abnormalities that can explain symptoms[1]. Diarrhea-predominant irritable bowel syndrome (IBS-D) is especially common in clinical practice. The pathogenesis of IBS is complex and has not yet been completely elucidated. In recent years, with the development of cell biology and molecular biology, growing evidence reveals that the occurrence and development of the disease are closely associated with tight junction structure damage of intestinal epithelial cells and the increased intestinal mucosal permeability. The process may be triggered by pro-inflammatory cytokine released by the activation of intestinal mucosal immune system, which directly acts on epithelial cell tight junction to down-regulate tight junction-related protein expression[2-4]. Tumor necrosis factor-α (TNF-α) may play a key role in the chain reaction of proinflammatory factor triggering tight junction structure damage[5,6]. On one hand, it can remodel cytoskeletal filament through Myosin light chain kinase (MLCK)-Myosin light chain (MLC) signaling pathway, leading to the increased contraction and tonicity of actomyosin ring in front of tight junction and causing the redistribution of Occludin, Claudin-1, Zonula occludens-1 (ZO-1) and other tight junction proteins and surrounding cytoskeletal proteins. As a result, the tight junction structure is shifted, the paracellular pathway is opened and the intestinal mucosal permeability is increased[7-9]; on the other hand, it can induce the production of Interleukin (IL)-β, IL-6, IL-8 and other pro-inflammatory factors, which form a cascade amplification effect[10]. The tight junction structure damage of intestinal epithelial cells is then strengthened. Metformin is a first-line drug in patients with type 2 diabetes mellitus, displaying a safe, effective, and total glycemic control effect in clinical practice in the past 50 years[11]. With the further research on hypoglycemic and other effects of metformin, the recent study indicates that the systemic inflammatory response can be inhibited by metformin[12,13]. Metformin can reduce the production of adenosine triphosphate (ATP) by inhibiting mitochondrial respiratory

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chain complex I. It can increase adenosine monophosphate (AMP)/ATP ratio by inhibiting AMP deaminase and reducing AMP clearance, resulting in activation of adenosine monophosphate-activated protein kinase (AMPK). The recent work reveals that metformin has a significantly inhibiting effect on inflammatory response after activating AMPK[14-16]. Metformin can inhibit the expression of cytokines in mouse macrophages stimulated by Lipopolysaccharides (LPS) in a dose-dependent manner. It also can inhibit the expression of adhesion molecules in endothelial cells activated by tumor necrosis factor-α (TNF-α) and the activation of nuclear factor kappa-B (NF-κB). This effect can be used to target at siRNA reversal of AMPK, suggesting that the anti-inflammatory effect of metformin depends on AMPK. Meanwhile, metformin can reduce the levels of inflammatory factors, IL-1β and IL-6[17-19]. Based on the above researches, we envisage whether metformin can regulate diarrhea-predominant IBS as well as potential mechanisms. Therefore, we carried out the current study to provide a potentially effective therapy for diarrhea-predominant IBS.

Materials and Methods

Materials

Anti-Occludin, anti-ZO-1, anti-Claudin-1, anti-MLCK, anti-MLC, anti-p-MLC and anti-β-catenin were purchased from Cell Signaling Technology (Danvers, MA, USA). The quantificational reverse transcription polymerase chain reaction (qRT-PCR) kit was purchased from Qiagen (Hilden, Germany), and other experimental kits were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The minimum essential medium (MEM) containing 10% fetal calf serum (FCS), 1% penicillin-streptomycin double-antibody fluid, and 1% non-essential amino acid, was selected as cell culture medium, which was cultured in an incubator (37°C, 5% CO_2).

Determination of Cell Transmembrane Resistance Value

The cells were inoculated on Transwell culture plate with the density of $8*10^4$/chamber and cultured for 14 days. Since the adding of TNF-α, the cell transmembrane resistance of cells in each group was measured at 2 h, 4 h, 8 h, 16 h and 24 h, respectively. The cell resistance was determined by EVOMX resistance meter (WPI Inc., Sarasota, FL, USA), and three points on each Transwell culture plate were randomly selected for determination. The changes in fluorescence yellow permeation rate of cellular macromolecules were determined. Cells in each group were treated by TNF-α for 24 h, and then the percentage of fluorescence-labeled macromolecule FD-4 through monolayer cell was measured.

Immunofluorescence

The cells were treated for 24 h and rinsed by phosphate-buffered saline (PBS) pre-heated to 37°C for 3 times. The transwell membrane was cut down by operating knife blade along the margin of chamber and fixed by 4% poly formic acid at 4°C; subsequently, it was rinsed by PBS for 3 times, 3 min each time, added with anti-ZO-1 (1:100) and Claudin-1 (1:100), respectively, and incubated at 37°C for 1 h. Next, it was rinsed by PBS for 3 times, 3 min each time, added with goat anti-rabbit IgG ALexa Fluor 488 (1:200) and incubated at room temperature avoiding light for 0.5 h. After rinsing by PBS for 3 times, 3 min each time, it was added with 4',6-diamidino-2-phenylindole (DAPI) and incubated at room temperature for 10 min. It was rinsed by PBS for 3 times, followed by mounting with buffered glycerin, and then it was observed, photographed and recorded under the confocal laser scanning microscope.

Western Blotting

The cells and grinding tissue were rinsed by precooling PBS and fully lysed by radio immunoprecipitation assay (RIPA) cell lysate, followed by centrifugation. The supernatant was taken. Through the protein quantification, 30 μg total protein sample was extracted and mixed with 5X sodium dodecyl sulfate (SDS) protein sample loading buffer, followed by denaturation at 100°C for 5 min loading in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The sample was treated by 350 mA electrophoresis. Then, the gel and activated polyvinylidene difluoride (PVDF) were placed onto transmembrane frame for constant current transmembrane for 2 h. PVDF membrane was taken and placed into 5% dried skimmed milk for sealing for 1 h. Subsequently, the membrane was added with primary antibody and incubated at 4°C overnight, followed by rinsing with Tris-buffered saline +

Tween 20 (TBST). Then, the membrane was added with horseradish peroxidase (HRP)-labeled secondary antibody and incubated at room temperature for 1 h, followed by rinsing with TBST and developing. ImageJ software was used to analyze gray level, and target protein/β-actin was adopted to represent relative expression of target protein.

Real-time Quantitative Polymerase Chain Reaction (PCR)

The total RNA of cell lines was extracted by TRIzol RNA kit, and the experimental procedure was conducted according to the instruction of kit. The concentration of total RNA was measured, and cDNA was synthesized by 1 μg RNA according to the instruction of reverse transcription synthesis kit. cDNA was used as a template to amplify and extract RNA in Coca-2 cell for reverse transcription-polymerase chain reaction. The primer sequences of myosin light chain kinase (MLCK): upstream primer: 5'-TGAGAGCCATTGCTAAGACTGT-3', downstream primer: 5'-TGAGAGCCATTGCTAAGACTGT-3'; the primer sequences of internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH): upstream primer: 5'-GGTCTCCTCTGACTTCAACA-3', downstream primer: 5'-AGCCAAATTCGTTGTCATAC-3'. The upstream and downstream primers of MLCK gene were treated by PCR amplification. The PCR was conducted on the Real-time quantitative PCR apparatus, and specific steps of reverse transcription and PCR amplification were performed in accordance with the instruction of kit. The relative expression of MLCK mRNA was calculated and analyzed by $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). p-values < 0.05 were considered statistically significant.

Results

TNF-α-Induced Epithelial Tight Junction Damage was Ameliorated by Metformin

The monolayer epithelial mucosal lesion model was established by Coca-2 cell and TNF-α to investigate the regulating effect of metformin on intestinal epithelial mucosal damage induced by TNF-α in this experiment. We adopted different concentrations of metformin (0.2 mM, 1 mM, 2 mM) to pre-treat well adherent monolayer Coca-2 cell for half an hour, and then 10 ng/mL TNF-α was added for the corresponding time, followed by detecting transepithelial electrical resistance (TEER) value of Coca-2 cell. These two indexes were utilized to investigate the regulating effect of metformin on epithelial tight junction damage, indicating that metformin can significantly inhibit TEER level induced by TNF-α (Figure 1A) in a certain dose-dependent manner. Further, the effect of metformin on TNF-α-induced Coca-2 cell permeability was explored, which was consistent with TEER result. Compared with the control group, the adherent cell permeability of Coca-2 cell was improved by TNF-α, while metformin inhibited the increase of TNF-α-induced Coca-2 cell permeability, displaying a certain dose-dependent manner (Figure 1B). The above results indicated that metformin can improve intestinal epithelial cell barrier induced by TNF-α.

Distribution and Expression of TNF-α-induced Epithelial Tight Junction-Related Protein was Ameliorated by Metformin

Intestinal epithelial tight junction is mainly regulated by the aid of related tight junction protein. TNF-α can disrupt the distribution of tight junction protein in cells and reduce its expression, thereby destroying the stability of tight junction. In order to investigate whether metformin has a stable effect on the abnormal distribution and expression induced by TNF-α, we adopted cell immunofluorescence technique to observe the regulating change of metformin on the abnormal distribution of ZO-1 and Occludin protein induced by TNF-α. The results of experiment (Figure 2) revealed that the fluorescence signal distribution of ZO-1 and Occludin protein in the TNF-α group was nonuniform, fluorescence intensity was reduced, and the joint was distinctly disrupted compared with the control group. The fluorescence signal intensity and junction in the metformin group were repaired, but none of them returned to the normal morphology compared with the model group. Based on this, it can be seen that the distribution damage and expression down-regulation of epithelial tight junction-related protein induced by TNF-α were ameliorated by metformin.

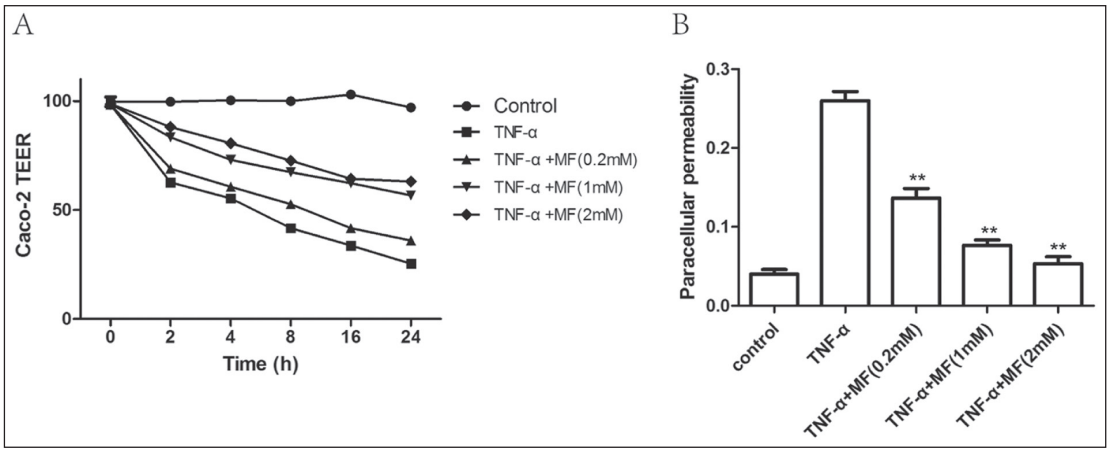

Figure 1. Metformin attenuates the effects of TNF-α on Caco-2 cell TEER and Paracellular permeability. Caco-2 cell monolayers were incubated with or without TNF-α (10 ng/mL) in the absence or presence of 0.2 mM, 1 mM, 2 mM metformin administered 30 min prior to TNF-a. TEER was measured at various time points. ***A,*** Caco-2 cell permeability was assessed using fluorescein permeability test at 24 hours after Caco-2 cell administered with TNF-α. ***B,*** Data shown are representative of 3 independent experiments. Data represented the mean ± SD (n = 3). *$p < 0.05$ *vs.* TNF-α group.

Inhibiting Effect of TNF-α on Epithelial Tight Junction-Related Protein Expression was Ameliorated by Metformin

Tight junction protein is the major functional protein of intestinal epithelial tight junction. The down-regulation of its expression can significantly affect its tight junction function, eventually resulting in the impaired intestinal epithelial barrier function. The fluorescence experiment showed that metformin can up-regulate the expressions of ZO-1 and Occludin protein in the TNF-α group. In order to further demonstrate it, we adopted Western blot method to detect the expression levels of ZO-1, Occludin and Claudin-1 protein intervened

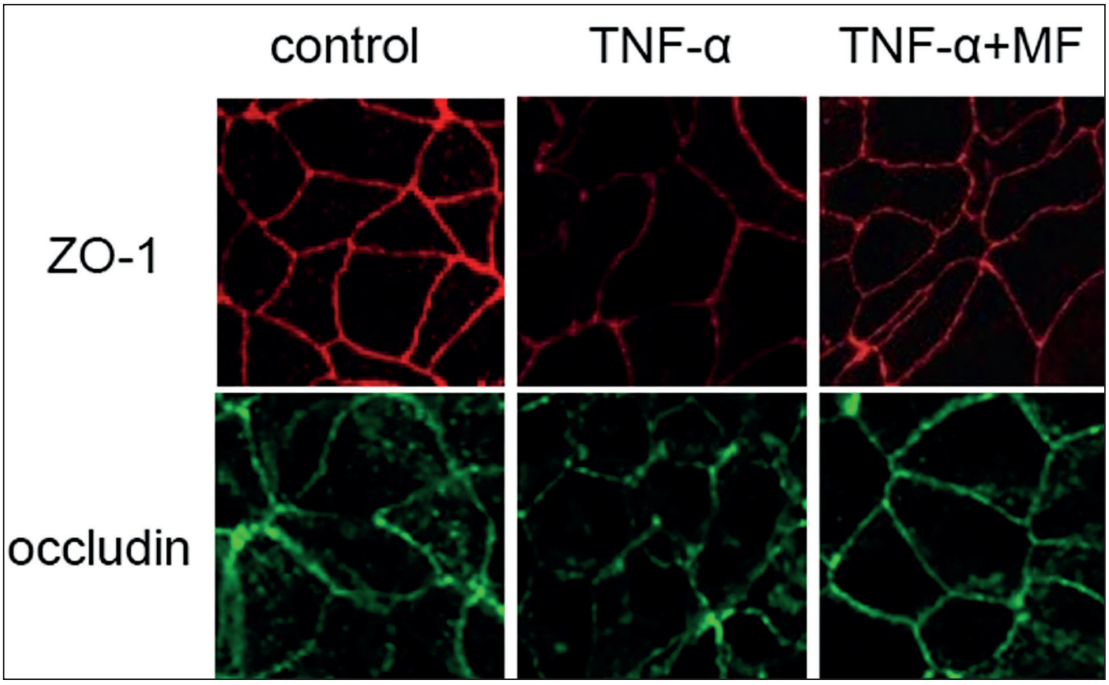

Figure 2. Metformin prevents TNF-α-induced morphological disruption of tight junctions. Caco-2 cell monolayers were treated with vehicle or TNF-α (10 ng/mL) in the absence or presence of metformin (1 mM) for 24 h and were then fixed and stained with anti-ZO-1 and occludin antibodies. Images were collected with a confocal laser-scanning microscope (1000×).

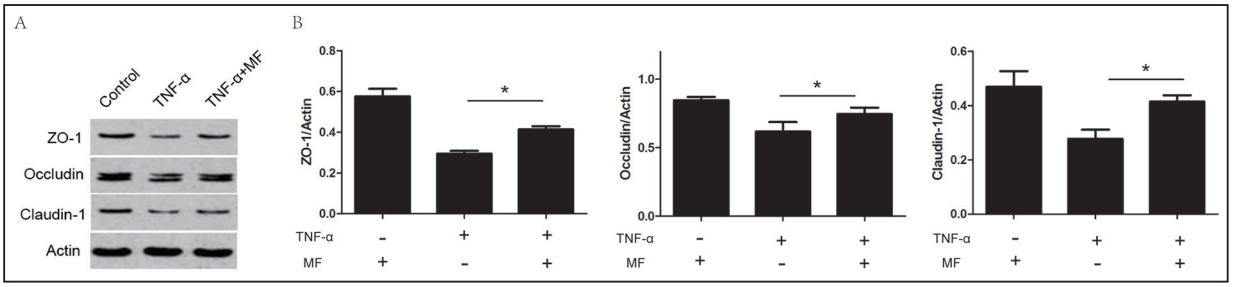

Figure 3. Effects of TNF-α and metformin on production of tight junction proteins. Caco-2 cell monolayers were treated for 24 h with vehicle or TNF-α (10 ng/mL) in the absence or presence of metformin (1 mM). The cell lysates were collected and immunoblotted for ZO-1, occludin, and claudin-1. ***A,*** β-actin served as the loading control. Relative protein density was calculated as the ratio of the protein density to the density of the vehicle. (B) Data shown are representative of 3 independent experiments. Data represented the mean ± SD (n = 3). **p* < 0.05 *vs.* TNF-α group.

by metformin. The experimental result (Figure 3) revealed that the expression levels of ZO-1, Occludin and Claudin-1 protein were down-regulated in the TNF-α group compared with the control group, which were up-regulated in the metformin intervention group than those in the model group. The result of Western blot further demonstrated that the inhibiting effect of TNF-α on epithelial tight junction-related protein expression was partially reversed by metformin.

Metformin Inhibited Up-regulation of TNF-α on p-MLC via Down-regulating MLCK Expression

Multiple studies have indicated that TNF-α can remodel cytoskeletal filament through MLCK-MLC signaling pathway, leading to the increased contraction and tonicity of actomyosin ring in front of tight junction and causing the redistribution of Occludin, Claudin-1, ZO-1 and other tight junction proteins and surrounding cytoskeletal proteins, which results in the shift of tight junction structure, the opening of paracellular pathway and the increase of intestinal mucosal permeability. Based on this, we envisaged whether metformin can ameliorate intestinal epithelial tight junction via regulating MLCK-MLC signaling pathway. Therefore, we adopted Real-Time PCR to determine the relative content of MLCK mRNA and detect the expressions of MLCK, MLC and p-MLC by Western-blotting. The result of Real-time PCR (Figure 4B) showed that the single metformin can inhibit mRNA expression of MLCK, show-

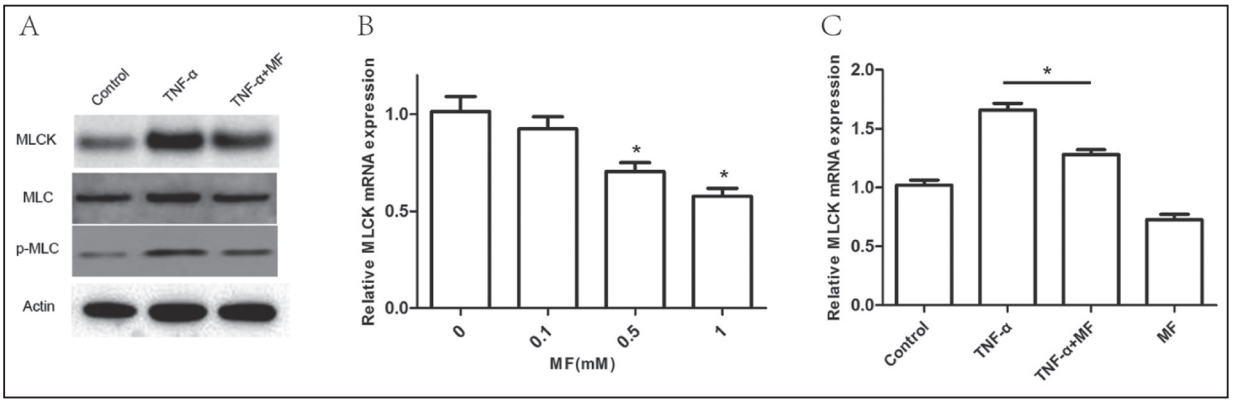

Figure 4. Metformin suppresses the TNF-α-induced increase in MLCK production. ***A,*** Caco-2 cell monolayers were treated for 24 h with vehicle or TNF-α (10 ng/mL) in the absence or presence of metformin (1 mM). Cell lysates were collected and subjected to Western blot assay for MLCK, MLC and p-MLC expression. ***B,*** Caco-2 cell monolayers were treated for 6 h with vehicle or 0.1, 0.5, or 1 mM rebeccamycin. Cell lysates were collected and subjected to qRT-PCR analysis for MLCK expression. ***C,*** Caco-2 cell monolayers were treated for 6 h with vehicle or 1 mM Metformin or TNF-α (10 ng/mL) in the absence or presence of metformin (1 mM). Cell lysates were collected and subjected to qRT-PCR assay for MLCK mRNA expression. Data shown are representative of 3 independent experiments. Data represented the mean ± SD (n = 3). **p* < 0.05 *vs.* control and TNF-α group.

ing a dose-dependent manner. In the combined experiment result, it could be seen that compared with the normal group, mRNA expression of MLCK in the model group was significantly increased, revealing that after TNF-α modeling can up-regulate mRNA expression of MLCK. Compared with the model group, mRNA expression of MLCK was distinctly decreased in the metformin group (Figure 4C). Being consistent with the result of Real-time PCR, the result of Western-blotting indicated that the expressions of MLCK, MLC and p-MLC in the model group were significantly up-regulated. Compared with the model group, the expressions of MLCK, MLC and p-MLC were down-regulated by metformin (Figure 4A). The above results showed that TNF-α can up-regulate MLCK-MLC signaling pathway expression, while metformin can significantly inhibit the expression in this pathway.

Discussion

The study indicates that cell tight junction structure and functional retention are correlated with the expression of tight junction protein. Cell tight junction main consists of Occludin, Claudin, Jams and other transmembrane proteins[20,21]. They constitute tight junction complexes with connecting complex proteins (ZO-1, ZO-2, ZO-3, etc.) and cytoskeletons (microtubules, microfilaments, silk, etc.). Transmembrane proteins are connected with connecting complex proteins and cytoskeletons. Various proteins with different structures, functions and mechanisms of molecular regulation, can be integrated by each signal transduction pathway to regulate the intestinal epithelial barrier function together[22]. Therein, alterations in the structure, function, and location of any protein can result in disruption of the tight junction structure and lead to the increased intestinal mucosal permeability[23].

In this research, the distribution and expression of transmembrane protein Occludin and connecting complex protein ZO-1 on cell membrane were observed by confocal laser scanning microscope, revealing that in cells of normal group, Occludin and ZO-1 protein are distributed along cell membrane, with close arrangement and smooth margin. After TNF-α was added for 24 h, the cell fluorescence intensity in the model group was reduced with interrupted phenomenon, indicating that the abnormal distribution of tight junction protein Claudin and ZO-1 was caused by TNF-α, which made it unable to fix at the tight junction point. Compared with the model group, the fluorescence intensity in the metformin group was enhanced, and the interrupted fluorescence signaling also was repaired, revealing that the shift of tight junction-related protein caused by TNF-α can be corrected by metformin at some extent. To further investigate the regulating effect of TNF-α on tight junction protein expression, we adopted Western-blotting to detect the expressions of Occludin, Claudin-1 and ZO-1 protein levels in each group. The results suggested that the expression levels were significantly reduced in the model group than those in the normal group. Compared with the model group, ZO-1 expression was up-regulated in the metformin group, showing a statistically significant difference, indicating that the expressions of connecting complex protein Occludin, Claudin-1 and ZO-1 can be down-regulated by TNF-α, while metformin can resist this effect. The previous study indicates that the tight junction structure change of intestinal epithelial cells caused by TNF-α is a rather complicated process[24,25]. There may be multiple signaling pathways involved, e.g., MLCK pathway, protein kinase C (PKC) pathway and mitogen-activated protein kinase (MKPK) pathway, which can regulate tight junction (TJ) structure via regulating tight junction proteins and assembly, dissociation and acidification of cytoskeletal proteins[22]. Therein, MLCK signaling transduction pathway exerts an important role in regulating the spatial conformation and function of cytoskeleton. Keita et al[25] found that the change of cytoskeletal structure induced by TNF-α is closely related to the activation of NF-κB, and further verified that the activation of MLCK transcriptional promoter is caused by TNF-α via NF-κB p65/p50 subunit acting on promoter downstream κB binding region. NF-κβ is a kind of nuclear protein factors widely existing in many tissues and cells. It has multi-directional transcriptional regulating effect, which can be involved in multiple gene transcriptional regulation after being activated. It also plays an important role in immune, inflammation, oxidative stress, cell proliferation, apoptosis and other physiological and pathological processes[26]. In the resting state, p65/p50 and inhibitor of NF-κB (IκB) in cytoplasm were synthesized to the trimer, so that p50/p65 cannot undergo

nuclear translocation. When cells are stimulated by inflammatory cytokines, endotoxin and other extracellular signals, inhibitor of nuclear factor kappa-B kinase (IKK) subunit is activated, causing the acidification and degradation of IκB. Then, p65 subunit is released from cytoplasm and transferred to the nucleus, specifically combining with IκB loci on gene, thereby regulating cell function[26]. TNF-α, as the extracellular signal, can activate NF-κB via a series of signal transduction pathways. The activated NF-κB p65 subunit is transferred from cytoplasm to the nucleus. It combined with the promoter of MLCK gene and triggered MLCK transcription, increasing MLCK protein expression. After MLCK activation, phosphorylation of eighteenth serine and nineteenth threonine in myosin light chain is further induced, and the conformation of MLC is changed. Phosphorylation of MLC can activate ATP enzyme in the myosin head, producing energy that causes cytoskeletal F-actin to slide. This leads to the increased contraction and tonicity of actomyosin ring in front of tight junction and causes the redistribution of Occludin, Claudin-1, ZO-1 and other tight junction proteins and surrounding cytoskeletal proteins, finally resulting in the shift of tight junction structure, the opening of paracellular pathway and the increase of intestinal mucosal permeability[27]. Xue et al[28] found that metformin can ameliorate the barrier function of the ileal epithelium in IL-10 gene knockout mice. The main mechanism is that activation of the AMPK signaling pathway by metformin can contribute to the differentiation of the intestinal epithelial stem cells of digestive tract, thereby improving the ileal epithelial barrier function in IL-10 gene knockout mice. Additionally, metformin can inhibit the aggregation of macrophages by lowering interferon γ (INF-γ) and TNF-α, and this mechanism also relieves inflammation and protects the intestinal mucosa. In order to investigate whether metformin is involved in other mechanisms in intestinal mucosal damage induced by TNF-α, we explored the regulating effect of metformin on MLCK-MLC signaling pathway. The results of Western-blotting and Real-time PCR consistently indicated that metformin can down-regulate the expressions of MLCK, MLC and p-MLC induced by TNF-α. Through inhibiting MLCK-MLC signaling pathway, the tight junction of intestinal mucosa is ameliorated by metformin, thus improving the intestinal mucosal barrier function.

Conclusions

Given that this study is limited to the cellular level *in vitro*, the research on the small intestinal barrier function at the animal level needs to be further explored to confirm its inhibitory effect *in vivo*. As a common clinical drug, the protective effect of metformin on the small intestinal mucosal barrier function provides a new perspective and a potential therapeutic approach for the treatment of inflammatory bowel disease, which still needs further basic and clinical studies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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